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UNSATURATED FATTY ACIDS REPRESS THE EXPRESSION OF ADIPOSE FATTY ACID-BINDING PROTEIN, aP2, IN RAW 264.7 MACROPHAGE

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by

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Adipocyte fatty acid binding protein, aP2, present in macrophages has been implicated in the integration of lipid metabolism and inflammatory response, contributing to development of insulin resistance and atherosclerosis. We investigated the modulation of aP2 expression by inflammatory insults and fatty acids in RAW 264.7 macrophages. When the cells were incubated with lipopolysaccharides (LPS; 100 ng/ml) or 10 ng/ml of tumor necrosis factor α for 18 h, aP2 mRNA and protein levels were drastically increased. Unsaturated fatty acids (100 μM of 18:1, 18:2, 18:3, 20:5 in complex with BSA), but not saturated fatty acids (16:0), significantly repressed the basal aP2 expression and abolished induction of aP2 expression by LPS. Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, increased aP2 mRNA levels but abolished the repressive effect of 18:2 on aP2 expression in unstimulated and LPS-stimulated macrophages. Cells transfected with siRNA targeting aP2 showed a relationship with cyclooxygenase-2 (COX-2), but not other pro-inflammatory mediators. In summary, our data suggest that unsaturated fatty acids may inhibit the basal as well as LPS-induced aP2 expression by mechanisms involving HDAC in RAW 264.7 macrophages and that there may be a link between COX-2 and aP2-repression by unsaturated fatty acids.

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Introduction

The link between lipid metabolism and inflammatory pathways has been suggested as a significant component in the development of insulin resistance, atherosclerosis, and type 2 diabetes (1-3). Dietary fat is an important macronutrient for the growth and development of all organisms (4). Excessive levels of dietary fat or an imbalance of saturated versus unsaturated fat have been implicated in the onset and progression of several chronic diseases, including coronary artery dieses, atherosclerosis, diabetes, obesity, and cancer (4). Specifically, unsaturated fatty acids are known to have positive health benefits such as reductions in atherosclerosis, inflammation, and plasma free fatty acid levels (5). However, the mechanisms for the anti-inflammatory properties of unsaturated fatty acids are poorly understood.

Fatty acid binding proteins (FABP) are implicated as players in the inflammatory response as well as lipid signaling cascades (2, 3). FABP are intracellular lipid chaperones that have a role in import, storage, and export of fatty acids because they reversibly bind hydrophobic ligands, such as saturated/ unsaturated long-chain fatty acids with high affinity (2).

The most characterized FABP is adipocyte fatty acid binding protein (aP2/FABP4), which is found in adipocytes at high levels and in macrophage cells to a lesser extent (2). Studies have shown that aP2 deficiency gives partial protection against atherosclerotic lesion formation in apolipoprotein E knockout mice, as well as, systemic insulin resistance, dyslipidemia, and lipotoxicity in *aP2*$^{-/-}$ *ob/ob* mice (2, 6).

The chronic low-grade inflammation associated with obesity is perpetuated by macrophage infiltration into lipid-engorged adipose tissue; this results in a release of various inflammatory cytokines (7, 8). Accompanying an obesity-induced inflammatory state are elevated circulating levels of aP2 (9).

The majority of research on the regulation of aP2 expression has been in adipocytes. Various cis-acting elements of the aP2 gene have been suggested, including an activator protein -1 (AP-1) sequence, and CAAT/enhancer-bind protein (C/EBP) binding element (10, 11). Recently it was demonstrated that macrophage aP2 can be regulated by phorbol 12-myristate 13- acetate, lipopolysaccharide, oxidized low-density lipoprotein and PPARγ ligands (12).

One way to affect gene expression is through the involvement of coactivator/ corepressor complexes (13). Histone deacetylases (HDAC) are essential components of co-repressor complexes as they provide the enzymatic activity for active repression(14). HDAC are a class of enzymes that remove acetyl groups from an ε-N-acetyl lysine amino acid on a histone. Its action is opposite to that of histone acetyltransferase (HAT) (15). Removal of acetyl groups gives chromatin a tightly-bound structure that prohibits transcription. Inclusion of acetyl groups decreases the affinity of histones for DNA and allows transcription to occur (15). There are eighteen known HDAC, which are divided into four classes. Class 1 HDAC are generally detected in the nucleus and ubiquitously expressed. Specifically, HDAC3 is unique among class 1 HDAC in that it can shuttle between the nucleus and cytoplasm and form complexes(15). Transcription factors that have been shown to interact with the corepressor complex for repressive effects on gene transcription include: AP-1 (16-18), nuclear factor κB (NF-κB) (16, 17, 19), liver X

receptor α (20), and thyroid hormone receptor (13, 21, 22). Upon activation, the co-repressor complex is exchanged with co-activators that lead to transcription (14).

Here we demonstrate histone modification involving HDAC as a mechanism for how fatty acids can affect aP2 expression in murine RAW 264.7 macrophage. Though therapeutic drugs are currently being developed to exploit aP2 for the treatment of diabetes and atherosclerosis (3, 12), the mechanisms for how aP2 expression is controlled under basal and activated conditions in macrophage is still unknown. Elucidation of aP2 regulatory mechanisms would allow for more accurate manipulation of pathways by dietary or pharmaceutical agents in order to positively influence health status.

Literature Review

Obesity and Inflammation

Obesity, which is defined as a body mass index (BMI) greater than or equal to 30 kg per m^2, predisposes an individual to develop insulin resistance, cardiovascular disease, diabetes, hypertension, high cholesterol, stroke, and certain cancers (23-25). The obesity epidemic reached a peak in 2003, and currently more than one-third of adults over age 20 in the United States are considered obese (24). Much of the complications associated with obesity are thought to be a product of the accompanying chronic inflammatory state in the adipose tissue (1, 7, 23, 25).

Adipose tissue is now being recognized as an endocrine organ, due to its ability to release pro- and anti-inflammatory protein factors, known as adipokines (26, 27). A chronic inflammatory response, characterized by elevated adipokine production and the activation of some pro-inflammatory signaling pathways, causes an induction of several biological markers of inflammation (28). These variations in gene expression of white adipose tissue are attributed to the infiltration of monocytes into lipid-engorged adipose tissue, which has been seen in both mouse models of obesity and obese humans (8, 28). It has been proposed that monocyte chemoattractant protein- 1 (MCP-1) (8), leptin (29), or activated endothelial cells (29, 30) could contribute to the macrophage infiltration into adipose tissue. Macrophages in the adipose tissue are responsible for most of the locally-produced cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6). TNFα is known to interfere with energy metabolism, especially lipid homeostatis by

increasing free fatty acid production (31). *In vitro* studies using adipocyte cell lines support the role of IL-6 as a factor to cause insulin resistance in adipocytes (32).

It is believed that the increases in local cytokine production from macrophage in lipid-engorged macrophages is the cause or possibly the consequence of the low-grade inflammation associated with obesity (28, 33, 34). The connection between adipose tissue and the immune system is clear, but why such a link exists is up for speculation. Coppack postulates that with the immune system using about 15% of resting metabolic rates, it would need to have a link to the availability of energy, especially the size of the adipose energy stores (35). This idea gives evolutionary purpose to the cross-talk between the immune and metabolic systems.

Inflammatory Signaling Pathways

Toll-like receptors (TLRs) play a critical role in the activation of the innate immune response in mammals by recognizing conserved pathogen-associated molecular patterns on invading bacteria (36). There are currently 13 known members of the TLR family in mammals, with TLR4 being a subclass (36). TLR4 can be activated by lipopolysaccharide (LPS), originating from the outer-membrane of gram negative bacteria, and non-bacterial agents such as saturated fatty acids (36, 37). The activation of TLR4 induces upregulation of intracellular inflammatory pathways such as c-Jun NH_2–terminal kinase (JNK) , nuclear factor –κB (NF-κB), and mitogen activated protein kinase (MAPK) pathways. The MAPK pathway can then lead to activation of the transcription factor activating protein 1 (AP-1) (36, 38).

TLR signaling in response to pathogen contact can contribute to the development of atherosclerosis (38). Loss of TLR4 leads to a protection against diet-induced obesity, I Kappa B Kinase β (IKKβ), and JNK expression, and insulin resistance (36). Activation of TLR4 by saturated fatty acids, not unsaturated fatty acids, can induce the expression of cycloxygenase-2 (COX-2), a marker for inflammation (39). Tsukuma *et al*. have suggested TLR4 may be a candidate for participation in insulin resistance induced by a saturated fatty acid-rich diet (36). It has been observed that saturated fatty acids can directly interact with the immune modulation and inflammation response through the activation of TLRs in macrophage (39).

Activation of the innate immune system can also be a result of TNFα. Binding of TNFα to its cell surface receptor induces the inflammatory response. TNFα is a major adipokine and its expression is highly induced in obesity (32, 35). It is known to interfere with insulin signaling after receptor binding on muscle cells or hepatocytes (32). TNFα causes an increase in free fatty acids, which encourage the development of insulin resistance in persons with metabolic disorders (31). Activation of a macrophage by either LPS or TNFα leads to downstream convergance onto similar inflammatory pathways. One route is through the IKK which signals the NF-KB pathway(40). Secondly, is through JNK and the AP-1 pathway (40).

Peroxisome Prolifeator Activator Receptor γ

PPARγ is a ligand-activated member of the nuclear receptor super family known to be involved in gene regulation of inflammatory and metabolic pathways (41, 42).

PPARγ was initially discovered as the master regulator in adipose cells (43). It is essential for the regulation of adipogenesis and is required for maintenance of mature adipocyte function, including triglyceride synthesis and storage (43-45). PPARγ is also expressed in activated monocytes/ macrophage and its agonist can inhibit the production of inflammatory cytokines by antagonizing the activities of transcription factors such as AP-1 and NF-κB (46, 47). One such antagonizing mechanism is through the ligand-dependant sumoylation of the PPARγ ligand-binding domain which inhibits the removal of a repressor complex from inflammatory gene promoters (14, 48). Alternatively, anti-inflammatory PPARγ activity can be inhibited by TNFα and this inhibition is thought to be involved in the pathogenesis of insulin resistance, atherosclerosis, and inflammation (6, 49). Naturally occurring ligands for PPARγ include long-chain fatty acids; other known synthetic PPARγ ligands include the thiazolidinedione class of anti-diabetic drugs (43). It is unclear as to whether aP2 enhances or inhibits the transcriptional activity of PPARγ, as experiments showing both have been reported (50, 51). A culmination of recent data suggests PPARγ agonists may regulate inflammation by the activation of PPARγ or alternatively through a PPARγ-independent mechanism (42, 44).

NF–κB Pathway

Production of pro-inflammatory cytokines is regulated largely by NF-κB (40, 42, 52, 53). It plays a pivotal role in the regulation over 100 genes, including TNFα, MCP-1, and cyclooxygenase 2 (COX-2) (54). In the canonical NF-κB pathway, p65 and p50 form a dimer and its activation is regulated by kappa B inhibitor protein (IκB) (14).

Following stimulation by inflammatory stimuli such as LPS, rapid proteasomeal degradation of IκB occurs after being phosphorylated by IκB kinase (IKK) (40). This allows NF-κB to shuttle from the cytoplasm into the nucleus, where it then promotes the transcription of target inflammatory genes (40).

Dietary agents have been shown to affect the NF-κB pathway. Fraxinellone, derived from root bark of *Dictamnus dasycarpus*, inhibits LPS-induced inflammatory response by negatively regulating NF-κB in RAW 264.7 macrophages (55). In contrast, *in vitro* culture of monocytes under high glucose condition compared to normal glucose led to the activation of NF-κB and significantly increased the expression of inflammatory cytokines including TNFα and MCP-1 (54).

MAPK and AP-1 Pathway

Upon binding of a peptide:MHC complex to a T-cell receptor, a cascade of proteins initiate the activation of the MAPK pathway (56). In the MAPK pathway, a three-kinase relay system, or MAPK cascade, involves three phosphorylation events that can lead to new gene transcription (56). The three most characterized groups of MAPK are extracellular signal-regulated kinases (ERK), p38 MAPK, and JNK (57). ERK are robustly activated by growth factors and phorbol esters but only weakly activated by cytokines and environmental stress (57). In contrast, p38 MAPK and JNK are strongly activated by cytokines and environmental stress but are poorly activated by growth factors and phorbor esters (57, 58). ERK works with the transcription factor Elk-1 to initiate transcription of Fos, a dimer component of AP-1 (56). JNK controls transcriptional activity of AP-1 via phosphorylation of c-Jun, allowing translocation of c-

Jun to the nucleus (56, 58). In the nucleus, c-Jun can dimerize with Fos to form AP-1 (56). In LPS-induced macrophages, phosphorylation of JNK was shown to initiate an inflammatory responses (59). Furthermore, JNK activity is significantly elevated in tissues of type 2 diabetic patients and in animals of obesity and diabetes (58).

AP-1 is a transcription factor that is a heterodimer of c-Jun, c-Fos, or activating transcription factor ATF proteins (60). It is activated by growth factors, neurotransmitters, cytokines, and bacterial or virus infections and is known to be involved in the transcriptional regulation of the inflammatory response (59).

Inflammatory Cytokines

Cytokines are a group of small, diverse proteins that are released by cells in the body in response to activating stimulus, such as pathogen invasion. They induce responses by binding to specific receptors and exert their effects in an autocrine, paracrine, or endocrine manner (35). The cytokines secreted by macrophage include: IL-1β, IL-6, IL-12, and TNF-α. IL-1β is responsible for activating vascular endothelium and lymphocytes (56). It is the cytokine responsible for the induction of fever (56). IL-6 is also responsible for lymphocyte activation and increases antibody production for the adaptive immune response (56). TNFα activates vascular endothelium and increases vascular permeability, which leads to increased entry of IgG, complement, and cells to tissues, and increases fluid drainage to lymph nodes (56). These cytokines are commonly used as experimental markers of an inflammatory response.

COX- 2 and Anti-Inflammatory Drugs

Cyclooxygenase (COX) catalyses the committed conversion of arachidonic acid to prostaglandin endoperoxide H_2 (PGH_2) and exists in two isoforms (61-66). These two isoforms of COX, i.e., COX-1 and COX-2, have different amino acid sequences but catalyze the same reaction. Both forms are membrane-bound, endoplasmic reticulum (ER)- resident, heme-containing glycoproteins that function as homodimers (63). COX-1 is constitutively expressed in most cell types (65, 66). In contrast, COX-2 expression is typically transient and is associated with inflammation (39, 64). The expression of COX-2 is a key element in various pathological conditions including arthritis, cardiovascular disease, and cancer (64).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that non-specifically target COX (61). They are prescribed for the treatment of inflammatory diseases such as arthritis and osteoarthritis. NSAIDs had initial success; however, long term therapy showed gastrointestinal (GI) toxicities due to the general inhibition of both COX-1 and COX-2. COX-1 expressed throughout the GI tract has protective effects on the GI mucosa by producing prostaglandin E2 (PGE2) (63). To avoid this side effect, research shifted toward selective COX-2 inhibitors. Selective inhibitors for COX-2, a class of drugs known as coxibs, show a decrease in inflammation. While, COX-2 knock-out mice have a marked protection from intestinal and skin tumors (66). Conversely, over-expression of COX-2 is observed in various malignant tissues (62). Depending on the cell type, COX-2 expression can be rapidly induced by the bacterial endotoxin LPS, cytokines such as IL-1, IL-2, and TNFα, and the tumor promoter, phorbol 12-myristate 13- acetate (PMA) (61). Though, even after stimulation, protein levels of COX-2 only

reach about 50% of COX-1 (61). After initial studies on COX-2 inhibitors, concerns arose regarding adverse cardiovascular events. Studies demonstrated that selective COX-2 inhibitors tip the natural balance between prothrombotic thromboxane A_2 and antithrombotic prostacyclin, which potentially increases the possibility of thrombosis (63). Research is on-going to find a selective COX-2 inhibitor that is not accompanied by severe side effects.

Fatty Acids

In the western diet, lipids represent more than 40% of the daily caloric intake, while nutritional advice recommends to limit lipid intake to 30% (26, 67). Fatty acids in the body can be exogenously brought in from the diet or can be generated from acetyl-CoA in a process called de novo lipogenesis (4, 68). A high fat diet is understood to greatly contribute to the increase of obesity prevalence as fatty acids are stored in fat cells composing adipose tissue (69). Carbohydrate (i.e. glucose) can be converted through de novo synthesis to fatty acids, which can also be stored in the form of triacylglycerol in adipose tissue (68).

Fatty Acids Metabolism

Mechanisms for the absorption of fatty acids differ according to the size and characteristic of the fatty acid. Long-chain fatty acids mix with bile acids in the intestinal lumen to form micelle particles (70). Micelles interact with the brush border of the intestine to allow fatty acid to diffuse from the micelle into the enterocyte, moving down

a concentration gradient (70). Fatty acids can also enter the cell through protein transporters, such as fatty acid transport protein (FATP) (71). From the enterocyte, fatty acids are packaged into chylomicrons that are then excreted into the lymph system (4, 69). Conversely, small-chain fatty acids have the ability to move directly from the intestinal lumen into portal circulation where they bind and are transported by albumin to the liver (70).

The liver and adipose are the two most important tissues in fatty acid metabolism. The liver is responsible for the uptake and catabolism of fatty acids as well as their repackaging into very low density lipoprotein (VLDL) in the form of triacylglercerol (70). The liver can also generate fatty acid from non-lipid precursors such as glucose (68). Fatty acids are delivered to adipose cells for storage. They can be delivered by VLDL, which would carry fatty acids originating from the liver, whereas dietary fatty acids are delivered to adipose cells by chylomicrons (70).

Fatty Acid Structure

The basic structure of fatty acids is a hydrophobic polycarbon chain which can vary in chain length with different degrees of saturation. Short- and medium-chain fatty acids, those with less than 6 or 14 carbons, respectively, are mainly saturated and used predominantly for energy supply. Long-chain fatty acids can be saturated as well as mono- or polyunsaturated, depending on the number of double bonds. They can serve functions apart from energy supply, including structural components of cells, gene regulators, involvement in cell growth and differentiation, or modulation of lipid, carbohydrate, and protein metabolism (4). The two major classes of long-chain

polyunsaturated fatty acids (PUFA) are ω-3 and ω- 6 fatty acids, where the ω-number indicates the position of the first double bond counted from the methyl group of the hydrocarbon chain. Current recommendations are to consume a higher percentage of ω-3 than ω-6 PUFA (72). Linoleic acid (18:2, ω-6) is an essential PUFA and the precursor for arachidonic acid (20:4, ω-6), while α-linoleinic acid (18:3, ω-3) is the precursor for eicosapentaenoic acid (20:5, ω-3) and docosahexaneic acid (22:6, ω-3). PUFA contribute roughly 7% of total energy intake in the diets of adults with linoleic acid (18:2, ω-6) being the most abundant in the diet (72). It has been established that fatty acids, specifically PUFA, can influence gene and protein expression (4, 26, 59, 73). Specifically, gamma- linoleic acid, which is converted in the body from linoleic acid by delta-6-desaturate, has the ability to inhibit the response generated by an inflammatory insult through inactivation of inflammatory pathways in RAW 264.7 macrophage (59). Unsaturated fatty acids, in general, are thought to have anti-inflammatory properties and many studies have given support to their inclusion in the diet (72, 74, 75).

Adipose Fatty Acid-Bindng Protein, aP2

Fatty-acid binding proteins (FABP) are cytoplasmic proteins with low molecular weight (~15 KDa) that bind fatty acids with high affinity in a 1:1 complex with an interior ligand binding domain (76). They are implicated as players in the inflammatory response as well as lipid signaling cascades (2, 3). FABP function as intracellular lipid chaperones with a role in import, storage, and export of fatty acids because they reversibly bind hydrophobic ligands, such as saturated/unsaturated long-chain fatty acids

with high affinity (2, 77). There are nine known FABP identified in different cell types (78). The most characterized FABP is adipocyte fatty acid- binding protein (aP2/FABP4) which is found in adipocytes at high levels and in macrophages to a lesser extent (2). Specific functions of aP2 other than a lipid chaperon are unclear; however, Thompson *et al.* (76) suggested that aP2 may act as a fatty acid sensor affecting cellular metabolism via protein-protein interactions as demonstrated by experiments involving Janus kinase 2 (JAK2) (76).

Mouse studies have shown that aP2 deficiency gives partial protection against atherosclerotic lesion formation in apolipoprotein E knockout mice, as well as systemic insulin resistance, dyslipidemia, and lipotoxicity in *aP2*$^{-/-}$ *ob/ob* mice (2, 6). Molecular mechanisms underlying the effect of aP2 on the pathogenesis of the diseases are still under investigation. aP2's involvement in these diseases may be, in part, from the ability of an inflammatory insult such as LPS to dramatically increase aP2 expression in RAW 264.7 macrophage; triglycerides in macrophage are also increased when treated with LPS (79). This paralleled increase suggests aP2 as an intermediary in the link between the immune and metabolism systems. Pharmacological agents specifically targeting aP2 are currently being investigated as a treatment for diabetes and atherosclerosis (3, 12).

An AP-1 sequence (80, 81), and C/EBP binding element (10, 11) have both been suggested as cis-acting elements of the aP2 gene. It was demonstrated that macrophage aP2 can be regulated by phorbol 12-myristate 13- acetate (PMA), LPS, oxidized low-density lipoprotein and PPARγ ligands (12). The mechanisms for how aP2 expression is controlled under basal and activated conditions in macrophage is still unknown.

Histone Modifications

Eukaryotic cell DNA is packaged into structures called nucleosomes (82). Each nucleosome is comprised of eight core histone proteins and the associated wrapped DNA (82). Histones are small, positively charged proteins and have close association with DNA. Eukaryotic cells commonly contain five abundant histone proteins: H1, H2A, H2B, H3, and H4 (82). H2A, H2B, H3, and H4 are considered the core histones because two copies of each form the protein core around which the nucleosome DNA is wrapped (82). H1 binds to the linker DNA and is referred to as a linker histone. Histone proteins tend to have a high concentration of positively charged amino acids, including lysine and arginine (82). By assembling into nucleosomes, DNA is approximately compacted 6-fold (82). The core DNA is a 147 base pair section that is wrapped around the histone octamer and then linked to the next histone octamer by a variable 20 – 60 base pair region of DNA (82).

Chromatin greatly impedes transcription. Epigenetic modifications of chromatin and DNA have been recognized as important permissive and suppressive factors in controlling gene transcription (83). Epigenetic mechanisms result in heritable modification in the expression of genes that are independent from DNA coding variability (83). Two major epigenetic mechanisms are the post-translational modification of histone proteins in chromatin and the methylation of DNA (83). Post-translational modifications of histones include, but are not limited to, lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation (84). Emerging evidence suggest a role of epigenetics in human pathologies, including inflammation (83). Additionally, it is known that nutrition and environment have the ability to influence the epigenetic profile.

Histone Deacytelases

One way to affect gene expression is through the involvement of coactivator/ corepressor complexes (13). Histone deacetylases (HDAC) are essential components of co-repressor complexes as they provide the enzymatic activity for active repression (14). HDAC are a class of enzymes that remove acetyl groups from an ε-N-acetyl lysine amino acid on a histone (15). Its action is opposite to that of histone acetyltransferase (HAT) which act as coactivators (15). Removal of acetyl groups gives chromatin a tightly-bound structure that prohibits transcription. Inclusion of acetyl groups decreases the affinity of histones for DNA and allows transcription to occur (15). There are eighteen known HDAC, which are divided into four classes (15). Class 1 HDAC are generally detected in the nucleus and ubiquitously expressed (15). Specifically, HDAC3 is unique among class 1 HDAC in that it can shuttle between the nucleus and cytoplasm and form complexes (15).

Transcription factors that have been shown to interaction with the corepressor complex for repressive effects on gene transcription include AP-1 (16-18), NF- κB (16, 17, 19), liver X receptor α (20), and thyroid hormone receptor (13, 21, 22). Upon activation, the co-repressor complex is exchanged with co-activators that lead to transcription (14). Using chromatin immunoprecipitation (ChIP), it was shown that high glucose conditions, similar to a diabetic state, could increase the recruitment of HAT to the promoter regions of inflammatory genes such as TNFα and COX-2 and lead to acetylation of nucleosomal factors histone H3 and H4 (54).

HDAC inhibitors are currently under investigation as anti-cancer agents because overexpression of HDACs has been observed in various cancers (15). Trichostain A (TSA) is a potent and nonselective inhibitor of HDAC (15, 85). TSA treatment has been seen to change the expression of only about 2% of genes (85). TSA is widely used to study the role of histone acetylation on gene expression.

Materials and Methods

Cell Culture and Fatty Acid Preparation

Murine RAW 264.7 macrophages (ATCC) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin/ 100 ng/ml streptomycin, 1x vitamins, and 2 mM L-glutamine in a humidified chamber at 37°C with 5% CO_2. Cells were plated at 5 x 10^5 per well in a 12-well plate to extract RNA and 1.5 x 10^6 per well in a 6-well plate for protein extraction. All cell culture supplies were purchased from MediaTech.

Two mmol/L of fatty acid-poor and endotoxin-free bovine serum albumin (BSA; Calbiochem) was prepared in phosphate buffered saline (PBS). Sodium salts of fatty acids (Nu-Chek) were dissolved in the 2 mmol/L BSA solution to a final concentration of 5 mmol/L. Fatty acids prepared included: myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). The fatty acid and BSA mixture was purged with N_2 , sonicated in a cool water bath until the solution became clear to form BSA/fatty acid complex (approximate molar ratio = 1:2.5), filter sterilized through a Millex®-GV 0.22 μM filter unit (Millipore), and diluted with cell medium to reach a final concentration of 100 μmol/L.

Raw 264.7 macrophages were incubated with BSA only (control) or 100 μM of a fatty acid for 6 h followed by BSA or fatty acid with 100 ng/mL LPS. For the inhibition of HDAC, RAW 264.7 macrophages were incubated in RPMI 1640 containing 500 nM TSA (Sigma-Aldrich) dissolved in dimethy sulfoxide (DMSO; Sigma-Aldrich) and 100

μM fatty acids for 6 hours followed by 14 hours in RPMI 1640 containing 500 nM TSA, 100 μM fatty acid, and 100 ng/ml LPS. For the experiments using the inhibitor, control cells were incubated with the same amount of DMSO vehicle. Macrophage activation was by LPS and TNFα. Cells were incubated with 100 ng/mL LPS (Sigma-Aldrich) or 2.5ng/ml, 5.0ng/ml, and 10ng/ml TNFα (eBioscience) for 18 hours and RNA was collected and analyzed by real-time PCR.

Small Interfering RNA (siRNA) Transfection

RAW 264.7 macrophages were transfected with Silencer® Negative Control siRNA (Ambion) (control) or Dharmacon siGenome SMART pool siRNA targeting aP2 (Thermo Scientific) for gene knockdown using DharmaFECT® 1 transfection reagent (Thermo Scientific) according to the manufacture's protocol. . In brief, 2.5 μl of transfection reagent DharmaFECT 1 was diluted into 97.5 μl of cell medium void of antibiotics and fetal bovine serum. Control and siRNA (2 or 3 μmol/L) were prepared in RNase-free sterile water and 50 μl of the diluted siRNA solution was mixed with 50μl of cell medium void of antibiotics and fetal bovine serum. Subsequently, the media containing transfection agent and siRNA were combined and incubated for 20 min at room temperature. The siRNA and transfection agent complex was then diluted to desired volume (1 ml). When recovering protein, reaction volumes were doubled and diluted siRNA concentration was 2.5μmol/L. Transfection occurred for 24 hours, after which cells were incubated with 100 μM fatty acids for 6 hours and then 100 μM fatty acid and 100 ng/ml LPS for 18 hours.

Total RNA Isolation and Quantitative Real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) following manufacturer's protocol or using the RNeasy Kit (Qiagen). Reverse transcription for cDNA synthesis and quantitative real-time PCR analysis were performed as previously described (86, 87). Primers were designed according to GenBank database using the Primer Express software (Applied Biosystems). The following primers were used for real-time PCR analysis: GAPDH, aP2, COX2, IL-1β, IL-6, and TNFα (Table 1).

HDAC Activity

Raw 264.7 macrophages were transfected with scrambled control or aP2 siRNA and incubated with 100 μM fatty acid for 6 h then 100 ng/ml LPS was added to fatty acids for an additional 18 h. Nuclear extracts were collected using Nuclear Extract Kit (Active Motif) according to manufacturer's protocol. Protein concentration was measured using BCA Protein Assay Kit (Thermo Scientific) and 10 μg of nuclear extract was analyzed for HDAC activity using a HDCA Activity Assay Kit (Cayman Chemical) according to the manufacturer's protocol.

Western Blot Analysis

Cell lysate was prepared and Western blot analysis was performed as previously described (88). Antibodies used for analysis were as follow: aP2/FABP4 (Santa-Cruz), and β-actin (Sigma-Aldrich). β-Actin was used as a loading control to normalize the data.

Statistical Analysis

ANOVA and Newman-Keuls Multiple comparison with Welch's correction for unequal variance when appropriate were used to identify statistically significant differences of treatments with $P < 0.05$ considered significant by GraphPad InStat 5 (GraphPad Software, Inc.). Data are expressed as mean ± SEM.

Table 1. Real-time PCR primers for mRNA expression

	Forward Primer 5’ → 3’	Reverse Primer 5’ → 3’
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
aP2	CACCATCCGGTCAGAGAGTACTT	CGGTGATTTCATCGAATTCCA
COX2	AAAGGTTCTTCTACGGAGAGAGTTCA	TGGGCAAAGAATGCAAACATC
IL-1β	GTCACAAGAAACCATGGCACAT	GCCCATCAGAGGCAAGGA
IL-6	CTGCAAGAGACTTCCATCCAGTT	AGGGAAGGCCGTGGTTGT
TNFα	GGCTGCCCCGACTACGT	ACTTTCTCCTGGTATGAGATAGCAAAT

Results

Expression of aP2 was induced by LPS stimulation in RAW 264.7 macrophages

Raw 264.7 macrophages were treated with 100 ng/ml LPS or 2.5 ng/ml, 5.0 ng/ml, and 10 ng/ml TNFα for 18 hours to evaluate the effect of an inflammatory insult on aP2 expression and quantitative real-time PCR analysis was conducted. LPS drastically increased aP2 mRNA abundance by ~8-fold compared with the control (Figure 1). Cells treated with TNFα also showed a ~2-fold increase in aP2 mRNA expression relative to the control (data not shown).

Unsaturated fatty acids (UFA) inhibited LPS-induced aP2 expression in RAW 264.7 macrophages

To investigate whether fatty acids could alter aP2 expression in response to an inflammatory insult, RAW 264.7 macrophage were incubated with fatty acids including myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) in the absence or presence of LPS. Consistent with previous data in Figure 1, LPS increased aP2 expression in the BSA control. Without LPS treatment, no significant difference in aP2 mRNA levels was seen in all the fatty acid treatment groups (Figure 2A). In contrast, when cells were activated by LPS, unsaturated fatty acids significantly decreased aP2 expression whereas saturated fatty acids did not alter the expression compared with the control. Western blot analysis confirmed the inhibition of LPS-induced aP2 protein levels by unsaturated fatty acids

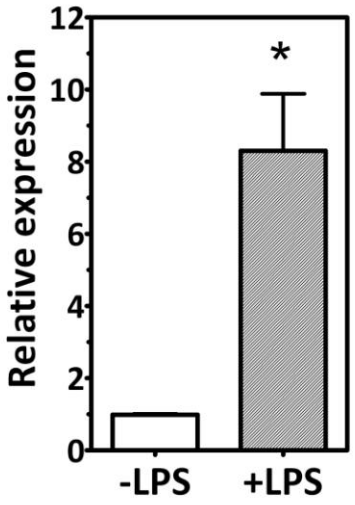
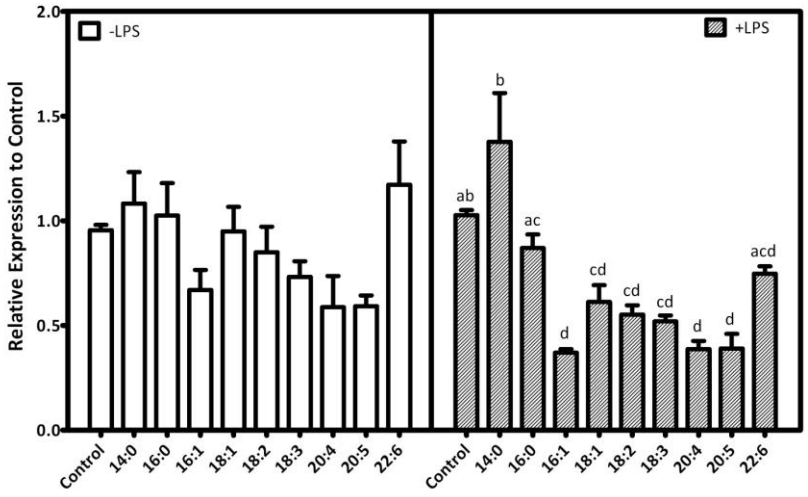


Figure 1. Elevated aP2 mRNA levels by LPS in RAW 264.7 macrophages. Cells were treated with 100 ng/ml LPS for 18 hours, after which quantitative real-time PCR was conducted to measure aP2 mRNA abundance. n = 9, Mean ± SEM. *, $P < 0.001$.

A



B

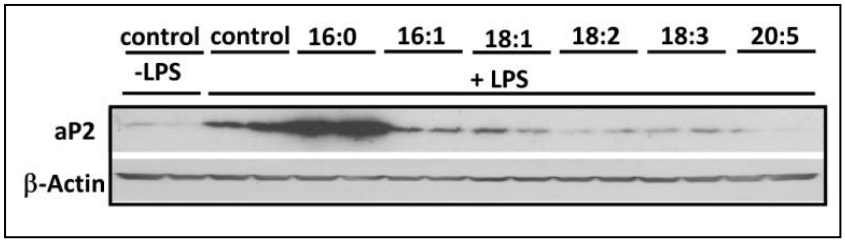


Figure 2. Elevated aP2 expression by LPS was abolished by unsaturated fatty acids in RAW 264.7 macrophages. Cells were treated with 100 μM fatty acid complexed with BSA for 12 hours and subsequently activated by 100 ng/ml LPS for 18 hours. A. aP2 mRNA expression was measured by real time PCR. n=3-4, Mean ± SEM. Bars with different letters are significantly different compared with control within the absence or presence of LPS treatment ($P < 0.05$). B. aP2 protein was measured by western blot analysis and β-actin was used as a loading control.

(Figure 2B). Palmitic acid markedly increased aP2 protein levels in the presence of LPS despite no significant change in mRNA levels.

TSA abrogateed UFA-mediated repression of basal and LPS-induced aP2 expression

To evaluate whether alterations in histone acetylation state is involved in the inhibitory effect of UFA on aP2 expression, RAW 264.7 macrophages were treated with TSA, an HDAC inhibitor (15), together with fatty acids, after which aP2 mRNA levels were quantified by real-time PCR. In consistent with our previous observations, LPS increased aP2 mRNA levels and 18:2 significantly lowered basal as well as LPS-induced aP2 expression in the absence of TSA (Figure 3). In the presence of TSA, however, the repressive effect of 18:2 on basal and LPS-induced aP2expression was abolished. The data indicate that 18:2 could inhibit basal and LPS-induced aP2 expression by modulating HDAC.

Palmitic acid decreased HDAC activity in RAW 264.7 macrophages

To evaluate the effect of fatty acids and aP2 on HDAC activity, Raw 264.7 macrophages were transfected with scrambled control or siRNA targeting aP2 for 24 h and then treated with 100 μM fatty acid for 24 h. aP2 protein levels were markedly diminished by siRNA in all the groups (Figure 4A). Compared to control, 16:0 significantly reduced HDAC activity regardless of aP2 deficiency (Figure 4B). Although knockdown of aP2 did not alter HDAC activity within each fatty acid treatment, HDAC activity was significantly elevated by 18:2 compared with control when aP2 was deficient.

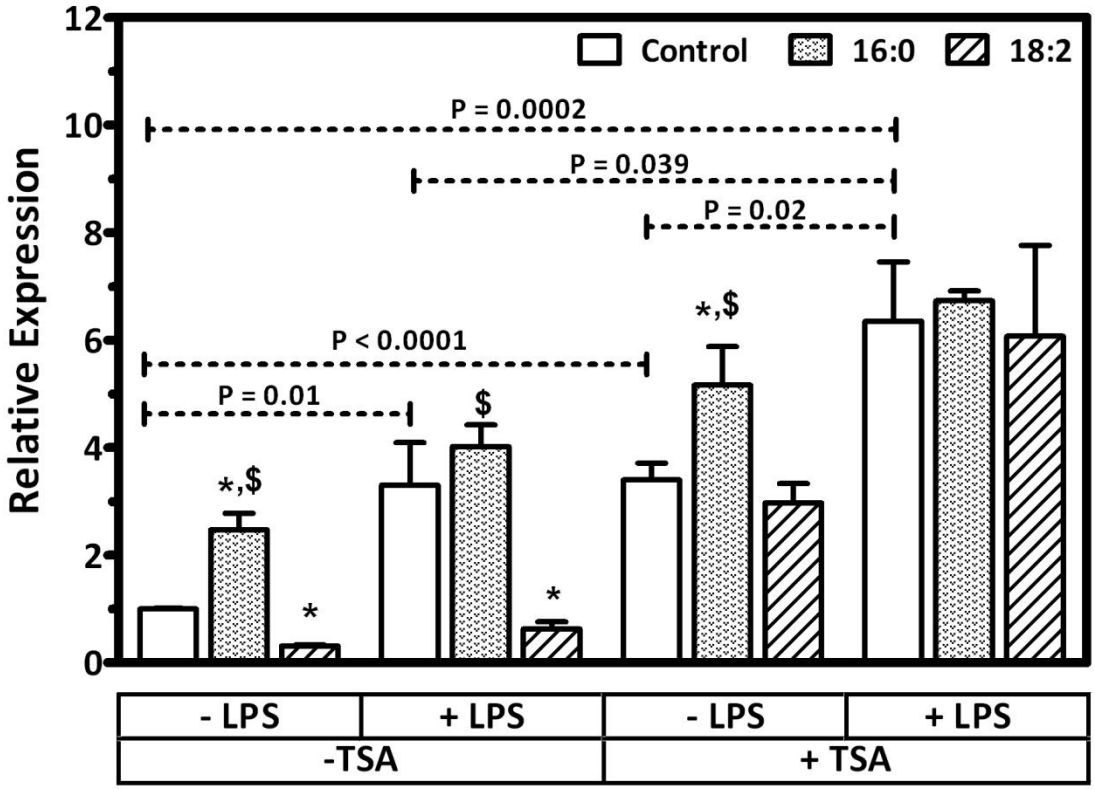


Figure 3. **Abrogation of repression of basal and LPS-induced aP2 mRNA levels by TSA in RAW 264.7 macrophages.** Cells were treated with 100 μM fatty acid and 500 nM TSA for 6 hours, after which 100 ng/ml of LPS was added for additional 18 hours. aP2 mRNA levels was measured by quantitative real-time PCR analysis. n=8-9, Mean ± SEM. *, P <0.05 compared with control in the same treatment. $^{\$}$, P < 0.05 compared with 18:2 in the same treatment.

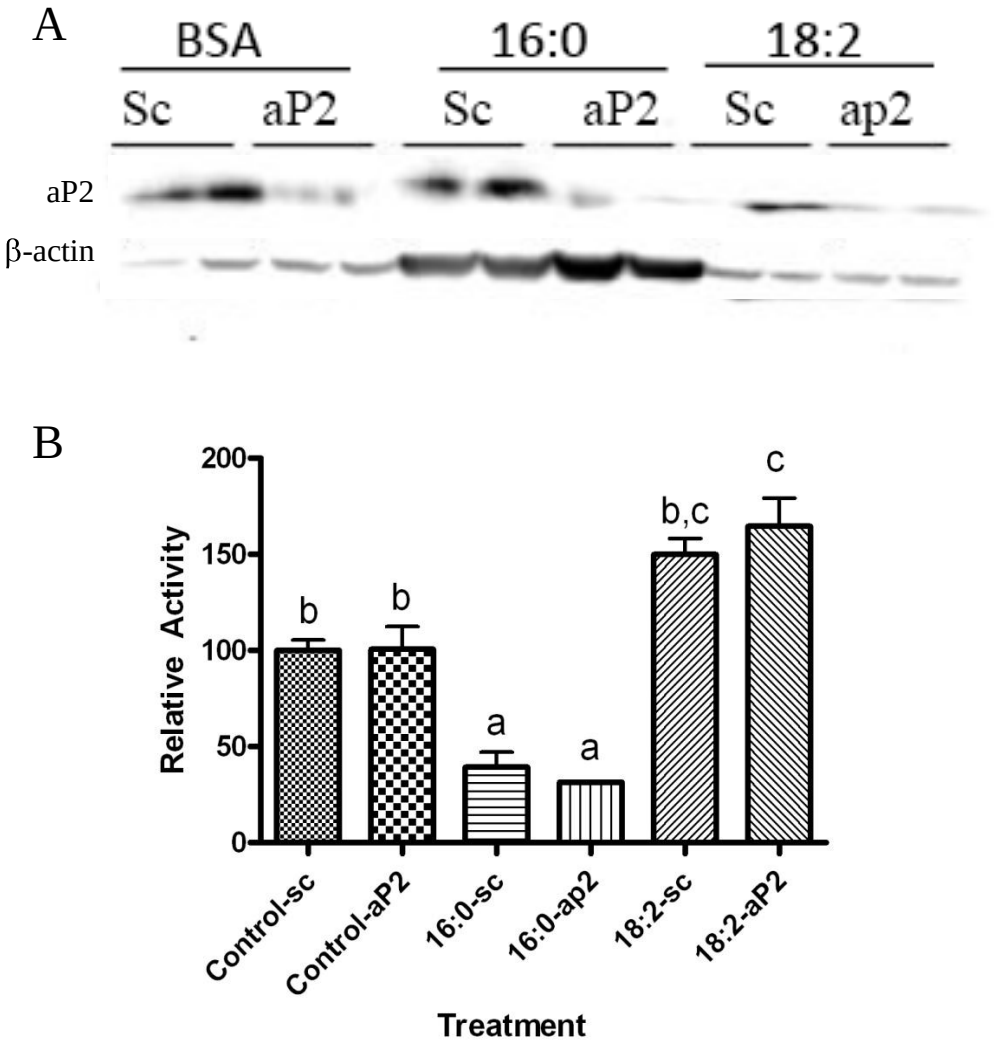


Figure 4. Inhibition of HDAC Activity by palmitic acid in RAW 264.7 macrophages. Cells were transfected with 125 nM of scrambled control (sc) or siRNA targeting aP2 for 24 h. They were subsequently treated with 100 μM fatty acid for 6 h after which 100 ng/ml LPS was added for additional 18 h. A. Nuclear extracts were collected to measure HDAC activity n = 4, Mean ± SEM. B. Cytoplasmic fractions were subjected to Western blot analysis to assess aP2 knockdown. β-actin was used as a loading control.

COX-2 was repressed by aP2 knockdown in LPS- stimulated RAW 2647 macrophages

To investigate whether aP2 plays a role in in the repressed pro-inflammatory gene expression by unsaturated fatty acids, RAW 264.7 macrophages were transfected with scrambled control or aP2 siRNA to knockdown aP2 expression. Cells were then treated with 100 μM of 16:0 and 18:2 for 6 h, after which 100 ng/ml LPS was added to cell culture medium to activate macrophages for 18 h. Real-time PCR analysis was performed to measure mRNA levels of aP2 and pro-inflammatory markers including IL-1β, IL-6, TNFα, and COX-2. aP2 mRNA levels showed a reduction by 75-80% by siRNA transfection (Figure 5) . In the unstimulated macrophages, deficiency of aP2 by siRNA increased the pro-inflammatory mediator expression and the increase was drastic particularly in 16:0-treated cells (Figure 6). In contrast, when cells were stimulated by LPS, aP2 knockdown had a minimal effect on IL-1β, TNFα and IL-6 mRNA levels. Of interest was that COX-2 mRNA abundance were significantly reduced when aP2 was deficient.

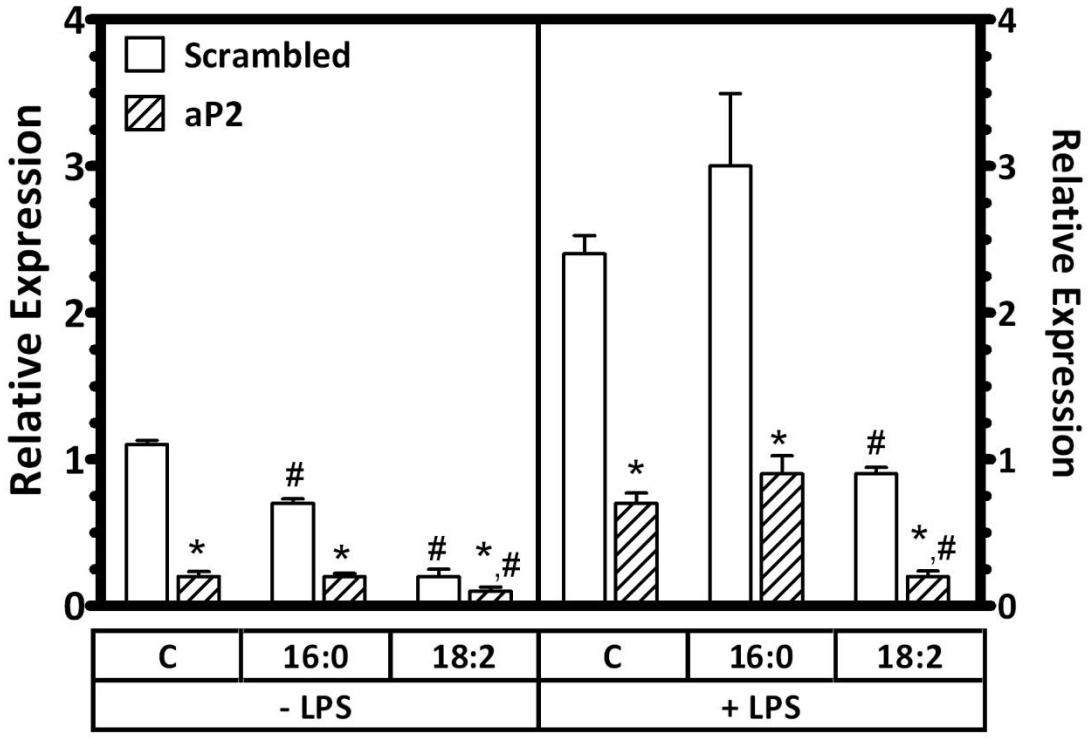


Figure 5. Knockdown of aP2 using siRNA in RAW 264.7 macrophages. Cells were transfected with 100 nM of scrambled control or aP2 siRNA and then treated with 100 μM fatty acid for 6 h. Subsequently, cells were activated by 100 ng/ml of LPS for 18 h. aP2 mRNA levels were measured by real-time PCR. n= 3, Mean ± SEM

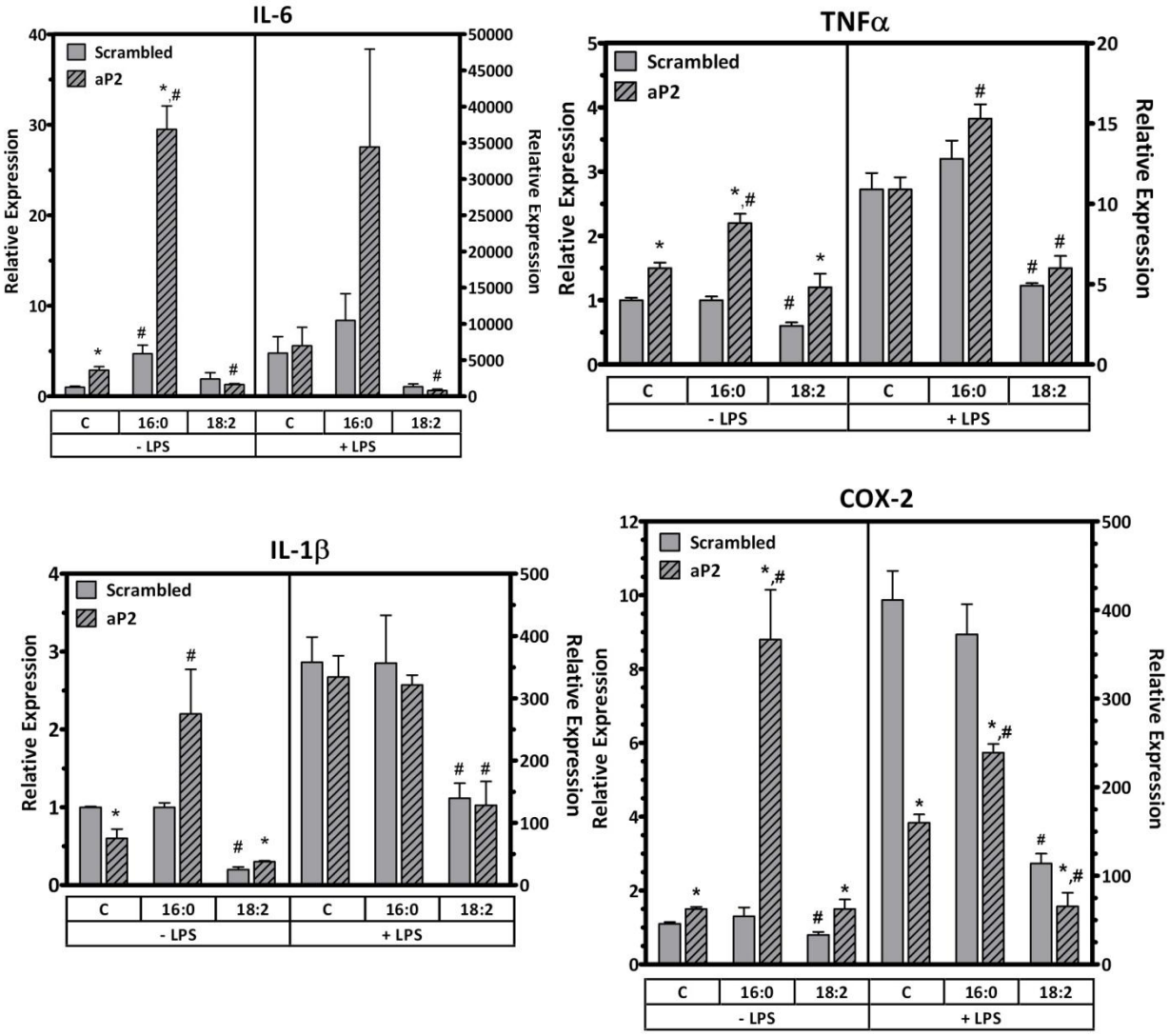


Figure 6. Effect of aP2 and FA on pro-inflammatory gene expression in RAW 264.7 macrophages. Cells were transfected with 100 nM of scrambled control or aP2 siRNA and then treated with 100 μM fatty acid for 6 hours. Subsuquently, cells were activated by 100 ng/ml of LPS for 18 hours. Expression of pro-inflammatory mediators including IL-6, IL-1β, TNFα and COX-2 were measured by real-time PCR. n = 3, Mean ± SEM.*, P <0.05 compared with scrambled control in the same fatty acid treatment. $^{\#}$, $P < 0.05$ compared with BSA control.

Discussion

aP2 deficiency in mice led the protection against the development of IR (89-91). Total and macrophage-specific aP2 knockout mice showed a marked reduction in atherosclerotic lesion formation in apolipoprotein E knockout (*apoE*$^{-/-}$) mice (92-95). In humans, a functional genetic variant of aP2 gene, resulting in reduced aP2 expression, showed a significantly lowered risk for Type 2 diabetes and CVD (96). Additionally, administration of an aP2 inhibitor in ob/ob and *apoE*$^{-/-}$ mice reduced the development of type 2 diabetesand atherosclerosis, respectively (12). The health benefits from absence or reduction of aP2 are linked to inhibited inflammatory mediator production in macrophages. We found that aP2 expression in macrophages is repressed by unsaturated fatty acids and acetylation of histones is likely to play a role in this repression. In addition, aP2 may contribute to the induction of COX-2 expression by inflammatory insults in macrophages.

In murine macrophage cell line, absence of aP2 markedly diminished pro-inflammatory cytokines (92). When THP-1 macrophages were incubated with the aP2 inhibitor, the production of pro-inflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1), IL-1β and IL-6 was significantly reduced (12). These studies suggest a significant role of aP2 in inflammatory signaling in macrophages. In the present study, both LPS and TNFα significantly increased aP2 expression in RAW 264.7 macrophages. Although cell surface receptors for LPS and TNFα are different, downstream mediators that can transduce their signals can converge onto similar pathways, including I Kappa B Kinases (IKK)/NF-κB and MAPK/AP-1 pathways.

Whether the induction of aP2 expression by LPS and TNFα is mediated through the pathways needs further investigation.

aP2 was initially thought to be controlled by PPARγ in adipocytes (97). It has been suggested by Genolet *et al.* (36) that PPARγ agonists, such as long-chain fatty acids, may regulate inflammatory pathways via both PPARγ-dependent and independent mechanisms. The observation that LPS increases aP2 in the present study is counterintuitive as LPS is known to inhibit PPARγ transcriptional activity (98). If PPARγ represents the major regulator for aP2 expression in macrophages, down-regulation of aP2 by LPS would be expected. Thus, another mechanism may exist by which LPS induces aP2 expression independent of PPARγ. AP-1 and C/EBP are shown to regulate aP2 expression in adipocytes and preadipocytes (80, 81, 99, 100). AP-1 is a more likely candidate for a transcriptional regulator of macrophage aP2 responding to inflammatory stimuli for the following reasons: first, AP-1 is one of the major transcriptional factors that are sensitive to inflammatory insults to produce pro-inflammatory mediators (101, 102); second, aP2 promoter contains AP-1 binding element; and third, LPS stimulation is shown to trigger signaling pathways leading to activation of MAPK (103-108) that can, in turn, activate AP-1. Therefore, MAPK/AP-1 pathway could be involved in the regulation of aP2 expression in macrophages.

Basal as well as LPS-induced aP2 expression in RAW 264.7 macrophage was repressed by unsaturated fatty acids but not by saturated fatty acids. Therefore, we sought to understand the mechanism for how unsaturated fatty acids can alter aP2 expression. Posttranslational modifications in histones such as acetylation, phosphorylation and ubiquitination are an important way to alter gene expression. Of the modifications,

histone acetylation is best understood. To gain an insight into a potential role of HDAC in the fatty acid regulation of aP2 expression, RAW 264.7 macrophages were treated with TSA. We observed that the repressive effects of the unsaturated fatty acids on the basal as well as LPS-induced aP2 expression were abrogated in the presence of TSA. The result is supportive to the idea that histone modifications, particularly acetylation, can be a mechanism by which fatty acids can alter aP2 expression in RAW 264.7 macrophages. Of interest is that basal aP2 expression was markedly increased by TSA regardless of the presence or absence of fatty acids suggesting that aP2 expression in RAW 264.7 macrophages is likely to be under active transrepression. Transcriptional factors namely AP-1, NF-κB and LXRα (17, 19, 109-111) have shown to exert active transrepression of gene expression through association with a co-repressor complex when they are not activated by their agonists. Nuclear receptor corepressors (NCoR) and silencing mediator for retinoid and thyroid receptor (SMRT) are essential components of a co-repressor complex and HDAC3 is a well-known HDAC isoform to interact with NCoR and SMRT (111-114). Therefore, we speculate that the co-repressor complex may be associated with an unknown transcription factor in the promoter of aP2 to repress basal gene expression in macrophages; and upon LPS stimulation, the co-repressor complex is dissociated to induce gene expression.

HDAC activity was measured to assess whether fatty acids alter the activity. Saturated fatty acids lowered HDAC activity. This observation is consistent with the observation that saturated fatty acids can increase transcription of inflammatory mediated such as COX-2 (39), in that a decrease in HDAC activity would coordinate with an increase in transcription. Furthermore, saturated fatty acids lowering HDAC activity is

also consistent with a trend seen in our data that saturated fatty acids can increase aP2 expression (data not shown). A similar parallel is seen with unsaturated fatty acids increasing HDAC activity. When macrophages were transfected with aP2 siRNA and treated with 18:2, there was a significant increase in HDAC activity. An increase in HDAC activity would coordinate with a repression in transcription. The trend toward significant for the negative control transfected cells treated with unsaturated fatty acid would suggest that unsaturated fatty acids are capable of affecting the HDAC activity. There was no significant differences in HDAC activity between the negative control and aP2 transected cells but this could be due to the fact the HDAC activity kit used measured universal HDAC activity and not a specific isoform. HDAC1 is the most abundance HDAC isoform in macrophage (data not shown), it is possible that fatty acids may not effect HDAC1 activity but could affect HDAC3 activity, which is the isoform believed to be part of the corepressor complexes that can affect transcription (115). An assay to measure universal HDAC activity would see HDAC1 overshadowing any effects that could be present in HDAC3. However, we speculate that HDAC3 as part of a co-repressor complex is influenced to continue or increase repression by unsaturated fatty acids but alternatively removed in the presence of saturated fatty acids. The absence of aP2 could further promote unsaturated fatty acids ability to influence the co-repressor complex.

In summary, we have shown a significant up-regulation of aP2 by an inflammatory insult, that unsaturated fatty acid repress both basal and induced aP2 expression, and that palmitic acid reduces HDAC activity in activated RAW 264.7 macrophage. This evidence suggests the involvement of HDAC and histone

deacetylation state in the mechanisms for how unsaturated fatty acids modulate a repression in LPS-activated macrophage. Lastly, a relationship between COX-2 and aP2 could be part of the mechanism for the anti-inflammatory effects of unsaturated fatty acids seen in macrophage. We have provided here new information on the mechanisms by which unsaturated fatty acids can regulate aP2 expression. Due to the high frequency of obesity in the United States, deciphering the mechanism for the anti-inflammatory properties of unsaturated fatty acid will allow for more appropriate and precise dietary interventions to prevent chronic metabolic and inflammatory diseases.

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